# Evaluation of High-throughput Genotoxicity Screening Assays Used in Profiling the 320 US EPA ToxCast™ Chemicals.

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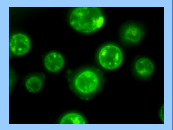
## Introduction

A key aim of the U.S. Environmental Protection Agency's ToxCast project is to investigate state of the art high-throughput screening (HTS) assays to provide a biologically informed basis for predicting toxicity and prioritizing chemicals for further testing[1]. The assessment of genotoxicity is a key element of the ToxCast project and an important component in toxicity risk assessment. Three HTS genotoxicity assays were used to analyze the collection of 320 predominantly pesticide active compounds in Phase I of the ToxCast research project: GreenScreen HC GADD45a-GFP (Gentronix Ltd.)[2], CellCiphr p53 activation (Cellumen Inc.)[3] and CellSensor p53RE-bla activation (Invitrogen Corp.)[4], the latter performed by the National Institutes of Health Chemical Genomics Center (NCGC). These assays each invoke components of cellular responses to a genotoxic challenge, which can lead to DNA damage, mis-repair, mis-segregation, mutations and, ultimately, to tumorigenesis and carcinogenesis. The assays measure 2 gene expression endpoints, the induction of p53 and the induction of GADD45a in a p53 competent cell line. p53 is known to act as a 'gate keeper' to ensure genetic and cellular integrity during the cell cycle. GADD45a (growth arrest and DNA damage) mediates the cell's response to genotoxic stress.

The HTS results were compared with comprehensive tumorogenicity data from the US EPA ToxRefDB database and, where available, historical mutagenicity (Ames) data. The protocols and performance characteristics of the genotoxicity assays have also been assessed as to their suitability for deployment in HTS campaigns. The aim of the present study was to evaluate the utility of HTS assays to identify potential genotoxicity hazard in the larger context of the ToxCast project, and to aid prioritization of environmentally relevant chemicals for further testing. The purpose of these HTS assays is not to replace the use of Ames and other established genotoxicity tests, but to increase testing efficiency to enable earlier and more efficient screening of larger sets of chemicals of interest.

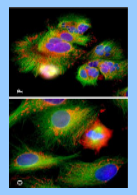
## The HTS – Genotoxicity Assays

### GreenScreen HC (GADD45a-GFP)



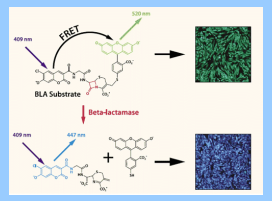
- TK6 human derived cell line with a GADD45a-GFP (green fluorescent protein) gene reporter. A second non-fluorescent strain is used to control for compound autofluorescence.
- Three serial dilutions of 200, 100 and 50 µM of each of 12 test compounds per 96-well microplate.
- Measurement at 24 and 48 hours by microplate spectrophotometer.
- Induction of cellular fluorescence is indicative of genotoxicity. Cytotoxicity is quantified by reduction in cellular proliferation, measured by optical absorbance.

### CellCiphr p53



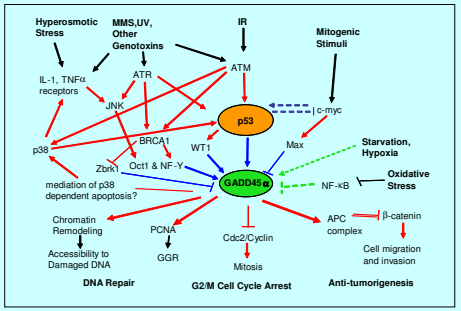
- The p53 assay is one of 10 cellular response endpoints in HepG2 cells.
- Nine serial dilutions from 200 µM for each of 16 compounds per 384-well microplate.
- Measurement of differential staining by a High Content Imaging platform.
- Employs an anti-p53 antibody.

### CellSensor p53RE-bla




- HCT-116 cells with stably integrated beta-lactamase reporter gene and p53 response element.
- 15 inter-plate dilutions from 92 µM for each of 1408 compounds over 15 1536-well microplates.
- Fluorescence resonance energy transfer (FRET) endpoint using a microplate spectrometer.

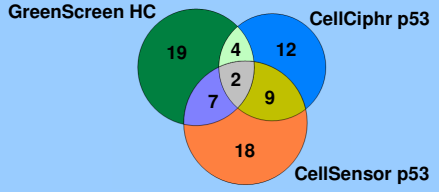
## p53 and GADD45α Cellular Pathways




Complex regulation of GADD45a and p53.

## Positive Results - and overlap between HTS assays

| Assay | Genotoxicity | | Cytotoxicity | |
|---|---|---|---|---|
| | Number | % | Number | % |
| GreenScreen HC | 32 | 10.4 | 231 | 74.8 |
| CellCiphr p53 | 27 | 8.7 | 171 | 55.3 |
| CellSensor p53 | 36 | 11.7 | - | - |




Number of compounds giving positive genotoxicity results in each HTS assay.

## Replicate Samples – Randomly distributed in the compound library

| Chemical Name | CAS Number | Replicate # | Source | Purity (%) | GreenScreen HC Genotoxicity | GreenScreen HC Cytotoxicity | CellCiphr p53 Genotoxicity | CellCiphr p53 Cytotoxicity |
|---|---|---|---|---|---|---|---|---|
| 3-Iodo-2-propynylbutylcarbamate | 55406-53-6 | 1 | Crescent | 97 | + + | + + | + | + |
| | | 2 | Sigma | 97.1 | + + | + + | + | + |
| Bensulide | 741-58-2 | 1 | Sigma | 99.5 | - | + + | - | + |
| | | 2 | Sigma | 99.5 | - | + + | - | + |
| | | 3 | Sigma | 99.5 | - | + + | - | + |
| Chlorsulfuron | 64902-72-3 | 1 | Crescent | 99 | - | - | - | - |
| | | 2 | Sigma | 99.9 | - | - | - | - |
| Dibutyl phthalate | 84-74-2 | 1 | Sigma | 99.63 | - | + + | - | + |
| | | 2 | Alfa Aesar | 98.6 | - | + | - | + |
| Diclofop-methyl | 51338-27-3 | 1 | Sigma | 99.2 | - | + + | - | + |
| | | 2 | Sigma | 99.2 | - | + + | - | + |
| | | 3 | Sigma | 99.2 | - | + + | - | + |
| EPTC | 759-94-4 | 1 | Sigma | 98.7 | - | - | - | - |
| | | 2 | Crescent | 97 | - | - | - | - |
| Fenoxaprop-ethyl | 66441-23-4 | 1 | Crescent | 98 | - | + + | - | + |
| | | 2 | Sigma | 98.4 | - | + + | - | + |
| Prosulfuron | 94125-34-5 | 1 | Sigma | 98.4 | - | - | - | - |
| | | 2 | Sigma | 98.4 | - | + | - | - |
| | | 3 | Sigma | 98.4 | - | - | - | - |

CellSensor p53 genotoxicity results were negative for all replicate samples.

## References and Links

(1) U.S. EPA National Center for Computational Toxicology ToxCast Project. www.epa.gov/ncct/toxcast.
(2) Knight A.W., Birrell L., Walmsley R.M., 2009. Development and validation of a higher throughput screening approach to genotoxicity testing using the GADD45a-GFP GreenScreen HC assay. 2009, Journal of Biomolecular Screening 14, 16-30.
(3) CellCiphr Cytotoxicity Profiling Assays. www.millipore.com/drugdiscovery/dd3/cellciphr
(4) Cell Sensor Cell Lines. www.invitrogen.com

## Key Results and Observations

- 9 – 12% of compounds were positive for genotoxicity in the HTS assays. The results of the three approaches only partially overlapped thus giving support to the proposed strategy of combining data from a battery of assays.
- The HTS assays were highly reproducible giving consistent results for replicate compounds.
- Overall the HTS assays demonstrated low sensitivity for rodent tumorigens likely due to: screening from a low, fixed concentration, coverage of selected genotoxic mechanisms, lack of metabolic activation in the assay protocols employed and difficulty in detecting non-genotoxic carcinogens.
- Conversely, HTS results demonstrated high specificity for rodent tumorigens (> 88%).
- The highest concordance with tumorogenicity data (>74% for HTS assays, c.f. 60% for Ames) was for compounds producing tumors in rodents at multiple sites and thus more likely genotoxic carcinogens.
- The highest 'positive predictivity' of multi-site rodent tumorigens was demonstrated by the GreenScreen HC assay (3 fold, vs. 1.41 – 1.65 for Ames and the HTS p53 assays).

## Concordance with Multiple Site Rodent Tumorogenicity Data

| | GreenScreen + | GreenScreen - |
|---|---|---|
| Rodent + | 13 | 45 |
| Rodent - | 16 | 199 |

| | CellSensor + | CellSensor - |
|---|---|---|
| Rodent + | 10 | 48 |
| Rodent - | 23 | 192 |

| | CellCiphr + | CellCiphr - |
|---|---|---|
| Rodent + | 8 | 50 |
| Rodent - | 18 | 197 |

| | Ames + | Ames - |
|---|---|---|
| Rodent + | 14 | 14 |
| Rodent - | 22 | 40 |

| | GreenScreen | CellSensor | CellCiphr | Ames |
|---|---|---|---|---|
| Number of comparisons | 273 | 273 | 273 | 90 |
| Sensitivity (% correct positives) | 22.4 | 17.2 | 13.8 | 50.0 |
| Specificity (% correct negatives) | 92.6 | 89.3 | 91.6 | 64.5 |
| Concordance | 77.7 | 74.0 | 75.1 | 60.0 |
| Balanced Accuracy | 57.5 | 53.3 | 52.7 | 57.3 |
| Relative Predicitivity (Positives) | 3.01 | 1.61 | 1.65 | 1.41 |
| Relative Predictivity (Negatives) | 1.19 | 1.08 | 1.06 | 1.29 |

## Comparison of Performance Characteristics

| | GreenScreen HC | CellCiphr p53 | CellSensor p53 |
|---|---|---|---|
| Endpoints | Genotoxicity / Cytotoxicity | Genotoxicity / Cytotoxicity | Genotoxicity |
| Microplate | 96 well | 384 well | 1536 well |
| Number of compounds / plate | 12 | 16 | 1408 |
| Number of dilutions / compound | 3 | 10 | 15 (in 15 microplates) |
| Incubation time / hr | 24 and 48 | 24 to 72 | 24 |
| Compounds per week (typical) | 720 | 100 - 500 | 30,000 - 100,000 |
| Equipment required | Conventional microplate reader (Tecan Infinite F500) | Image analysis equipment (ArrayScan) | Microlitre liquid handling automation on ultra-high throughput robotic platforms |
| Data interpretation | Straightforward and automated - using proprietary Excel software template | Specialist image analysis software - 1 of 10 endpoints therefore data extraction required | Straightforward and automated - using proprietary software. Some user input to identify artifacts |
| Reagents | Proprietary cell lines and media from Gentronix | Proprietary cell lines and media from Millipore | Proprietary cell lines and media from Invitrogen |

## Concluding Remarks

Data from these HTS genotoxicity assays alone are not proposed as surrogates for the comprehensive regulatory genotoxicity assays or as accurate predictors of animal tumorogenicity. Rather data from the HTS assays can be applied in the following areas within the wider context of the aims of the ToxCast project:

- High-throughput screening for compounds capable of causing genetic damage *in vitro* and thus for 'hazard identification', i.e. highlighting chemicals with the potential to induce carcinogenicity.
- As part of a weight of evidence assessment of the likelihood of a compound's adverse effect for humans.
- To help determine the mode of action for carcinogenicity.
- To aid prioritisation of a compound for follow up *in vitro* and *in vivo* testing.